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**13. SUPPLEMENTARY NOTES**

**14. ABSTRACT**
Despite improved treatments, metastatic breast cancer kills more than 40,000 women each year in the US. Little is known about what factors in the host contribute to the establishment of metastases. To understand how the host microenvironment affects the behavior of cancer cells, we have used the zebrafish, a powerful, genetically tractable vertebrate model system with cancer biology very similar to human. Our studies focus on the interaction of the chemokine receptor CXCR4b, expressed in breast cancer cells, with its ligand sdf-1, expressed in the microenvironment. This interaction is a key determinant of metastatic potential. We have selected breast cancer lines with varying expresssion levels of CXCR4b and sdf-1. We have selectively altered the expression of sdf-1 in zebrafish embryos using knockdown and targeted expression techniques. As proof-of-principle, we shown that perturbing embryo sdf-1 directly affects the migration of endogenous primordial germ cells, which also depend on CXCR4b. In subsequent work, we will assess the effect of these manipulations on survival and spread of breast cancer xenografts. The goal of this study is to understand how the host microenvironment influences the development of breast cancer metastasis.

**15. SUBJECT TERMS**
breast cancer, metastasis, chemolkine receptor, microenvironment, zebrafish, xenograft

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## INTRODUCTION

Despite improved treatments, metastatic breast cancer kills more than 40,000 women each year in the US. Analysis of metastatic breast cancer cell lines has revealed many factors produced by the tumor cells that promote metastasis, for example matrix metalloproteinases, adhesion molecules and angiogenic growth factors. In contrast, relatively little is known about what factors in the host contribute to the establishment of metastases. Recent intriguing evidence points to novel mechanisms, such as alphavbeta3 integrin expression on endothelial cells, expression of chemokine CXCL12 by target tissues and p53 mutations in tumor-associated stroma, as contributors to invasiveness and metastatic potential (Kurose, Gilley et al. 2002; Dewan, Ahmed et al. 2006; Gupta and Massague 2006). It is critically important to discover other such host factors; however the tumor microenvironment has been difficult to study because it cannot be manipulated as facilely as can the cell lines. To address this problem we have turned to the zebrafish, a powerful, genetically tractable vertebrate model system with cancer biology very similar to human (Amatruda, Shepard et al. 2002). The goal of this study is to understand how the host microenvironment influences the development of breast cancer metastasis.

We hypothesize that, when placed in zebrafish embryos as xenografts, human breast cancer cells will recruit host factors to survive, proliferate and spread. Recently, it was shown that human melanoma cell xenografts survive and stimulate angiogenesis when placed in zebrafish embryos (Haldi, Ton et al. 2006). The overall approach of this project is to use knockdown and mutagenesis techniques in zebrafish embryos to systematically alter the tumor microenvironment, assessing the impact on growth and invasiveness of the xenografted human breast cancer cells.

## BODY:

In the first phase of this project, we chose to focus on the role of chemokine signaling in the interaction of breast cancer cells with their environment. The chemokine receptor CXCR4b is expressed in breast cancer cells and is associated with tumor growth, spread and angiogenesis (Dewan, Ahmed et al. 2006; Liang, Brooks et al. 2007). The ligand for CXCR4b is known as CXCL12 or Sdf-1a (for Stromal-derived factor-1a). Our goals was to create an experimental system whereby we could investigate the role of Sdf-1a expression in the microenvironment. Specifically, we set up a system to manipulate Sdf-1a expression in zebrafish embryos. The experimental scheme is shown in **Figure 1**. Using a robust antisense technology known as a morpholino, we can knock down endogenous *SDF-1* expression; this approach leads to stable knockdown for 4-5 days. Separately, we inject an embryo with mRNA encoding zebrafish Sdf-1a along with a fluorescent label. We then transplant labeled cells expressing high levels of *SDF-1* into the knockdown embryos. This creates a localized area of *SDF-1* expression. The purpose of this proof-of-principle experiment is to assess whether misexpression of Sdf-1a is sufficient to alter the migration of Primordial Germ Cells (PGCs). To more easily visualize PGCs in living animals, we take advantage of the of *askopos*:GFP transgenic line, in which the PGCs are tagged with GFP. We then assess whether PGCs (green) migrate toward the *SDF-1*-expressing cells (red).

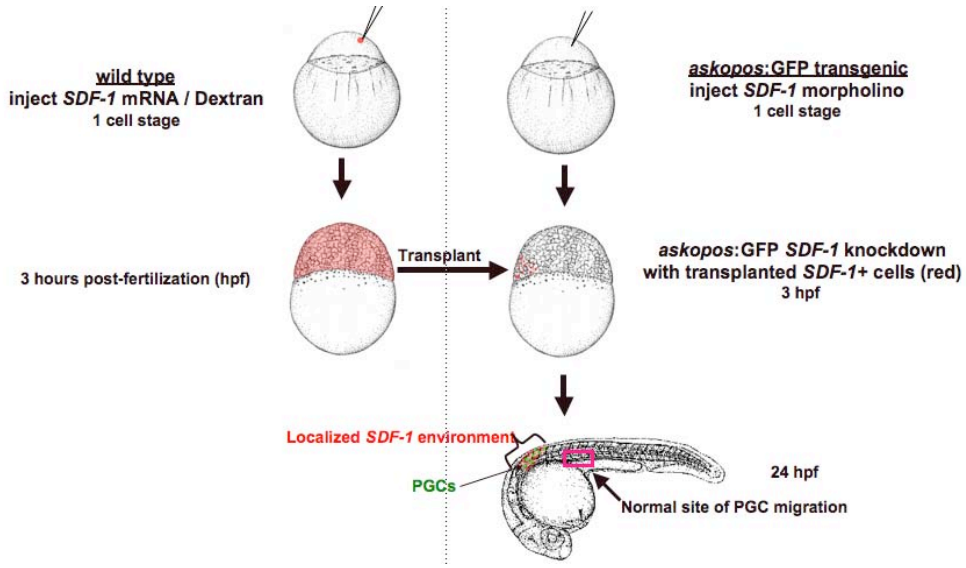


**Figure 1: Modeling the *CXCR4/SDF-1* Interaction: Primordial Germ Cell Migration.** In zebrafish, *CXCR4* expressed in PGCs responds to *SDF-1* expressed in somatic cells to guide PGC migration. In our approach, we first knock down endogenous *SDF-1* expression with a morpholino, then transplant labeled cells expressing high levels of *SDF-1* into the knockdown embryos. This creates a localized area of *SDF-1* expression. Use of *askopos*:GFP transgenic embryos allows visualization of migrating PGCs in the live embryo. We then assess whether PGCs (green) migrate toward the *SDF-1*-expressing cells (red).

To demonstrate successful knockdown, we injected Sdf-1a morpholino into *askopos:*GFP transgenics and assessed PGC migration. **Figure 2** shows that knockdown of Sdf-1a leads to aberrant migration of PGCs.

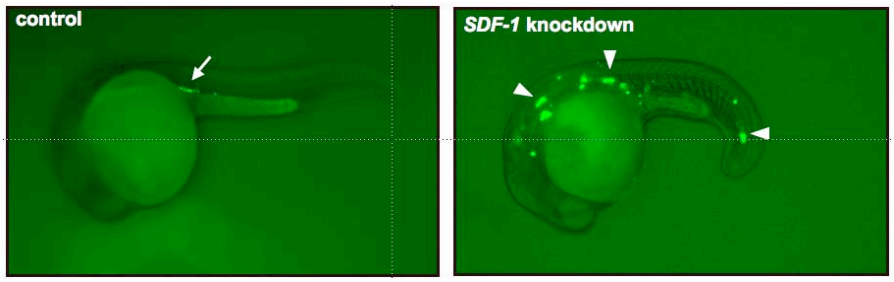


**Figure 2. Knockown of Sdf-1a Perturbs PGC Migration. Control** embryo demonstrates normal clustering of PGCs in presomitic mesoderm (arrow).
***SDF-1* knockdown** by morpholino injection causes ectopic PGCs (arrowheads).
Methods: Single-cell stage *askopos*-GFP transgenic zebrafish embryos were injected with control or *SDF-1* morpholino. PGCs were visualized 24 hours post-fertilization with epifluorescence microscopy.

We then proceeded to carry out the experimental scheme outlined in Figure 1. **Figure 3** shows an embryo in which the GFP-labeled PGCs have ectopically migrated to sites of high Sdf-1a expression, as identified by Texas Red dextran-labeled transplanted cells.

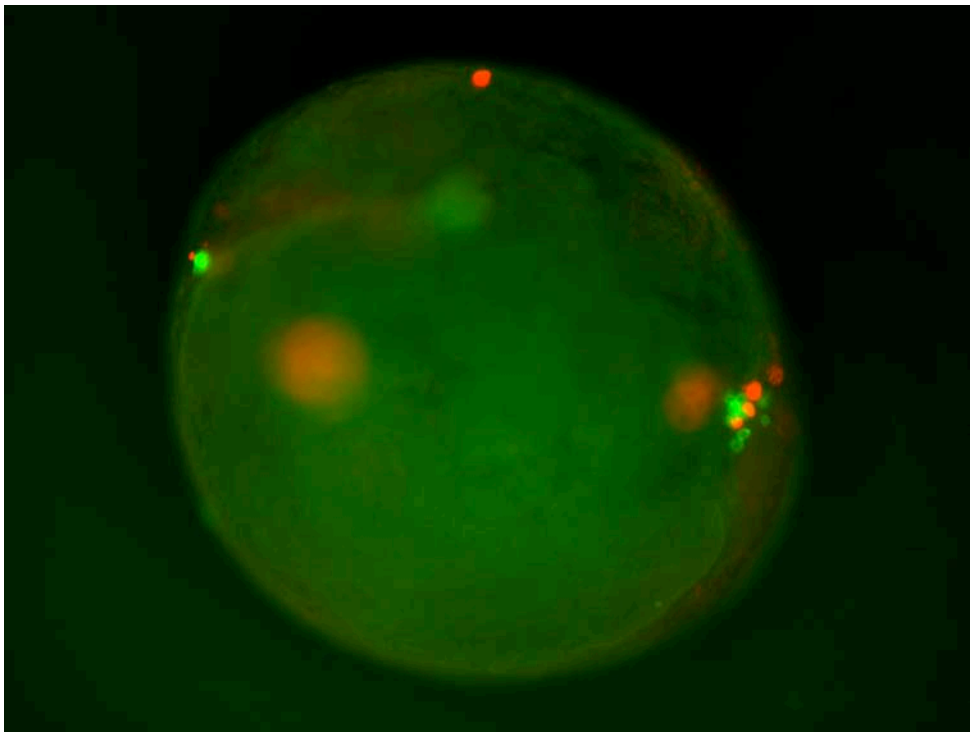

**Figure 3. PGCs migrate ectopically toward misexpressed Sdf-1a.** PGCs (green) in an *askopos*-GFP *SDF-1* knockdown embryo migrate toward exogenous cells (red) expressing high levels of *SDF-1*.
Methods: 1-cell stage *askopos*-GFP transgenic zebrafish embryos were injected with an *SDF-1* morpholino. 1-cell stage wild-type zebrafish embryos were injected with *SDF-1* mRNA and Texas Red Dextran. The *SDF-1* positive cells were transplanted into the *askopos*-GFP *SDF-1* knockdown embryo at 3 hours post-fertilization, creating localized *SDF-1* expression. Cells were visualized 24 hours post-fertilization with epifluorescence microscopy.

A key aspect of the experimental scheme is showing that we can transplant human tumor xenografts into zebrafish embryos, and the cells will survive and be visible when fluorescently labeled. **Figure 4** demonstrates labeled human tumor xenografts in live zebrafish embryos. We also went on to characterize CXCR4b and Sdf-1a expression in a large collection of primary breath cancer cell lines at UT Southwestern Medical Center. Currently, we are growing these cells and attempting to achieve stable, fluorescently-labeled cell lines from each. Once the lines are obtained, we will carry out the experiment to observe the effect of Sdf-1a misexpression on human breast cancer cell proliferation and migration (experimental schema shown in **Figure 5**.)

**KEY RESEARCH ACCOMPLISHMENTS**

- We have generated a system whereby we can selectively alter a key component of the tumor microenvironment (Sdf-1a expression)
- We have shown that misexpressed Sdf-1a in the embryo microenvironment serves as a guidance cue for PGC migration, which depends on CXCr4b/Sdf-1a interaction.
- We have demonstrated that labeled human tumor cells can be xenografted into zebrafish embryos
- We have identified breast cancer cells lines with varying expression of CXCR4b and Sdf-1a expression levels
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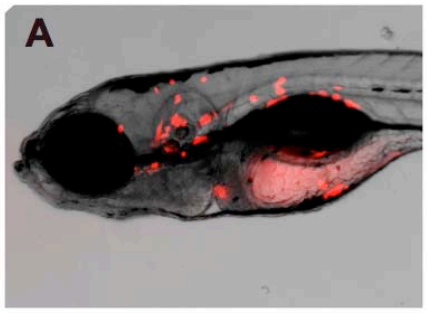


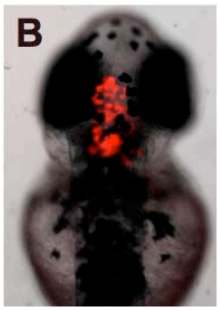


C

| Cell Lines | *CXCR4* expression | *CXCL12 /SDF-1* expression |
|---|---|---|
| HCC70 | low | low |
| HCC1257 | high | low |
| HCC1500 | high | high |

**Figure 4. Human tumor xenografts survive in zebrafish embryos. A-B**, labeled human tumor cells (red) xenografts were placed in zebrafish embryos at 3 hpf (**A**) or into the neural tube at 24 hpf (**B**) and imaged at 72 hpf. Human tumor cells survive at least 7 days in zebrafish embryos. **C**, gene expression analysis of primary human breast cancer cell lines was carried out using Affymetrix HG-U133A and B. Lines were selected for xenografts based on *SDF-1* and *CXCR4* expression.

**REPORTABLE OUTCOMES:**

**Abstract** submitted to 2008 Era of Hope Meeting, Baltimore, MD
**Poster Presentation** 2008 Era of Hope Meeting, Baltimore, MD

**CONCLUSION:**

We have taken the important initial steps into creating a genetically-tractable model of the tumor microenvironment, focusing on the critical CXCR4b/Sdf-1a interaction. Importantly, we were able to use endogenous Primordial Germ Cell Migration as a reporter to show that we successfully modified Sdf-1a expression in a way that reveals CXCR4b-meidated migratory events. We now need to repeat the experiment using labeled human breast cancer cells as the reporter. Once successfully established, this model could be used as a rapid, convenient and physiological model of CXCR4b-dependent breast cancer cell migration. The main

purpose of such a model would be to test molecular hypotheses (e.g. by knocking down key target genes in breast cancer cells, or by knocking down or misexpressing genes in the embryo microenvironment) or for relatively high-throughput screening or novel agents that could inhibit breast cancer cell migration and therefore potentially abrogate metastasis.

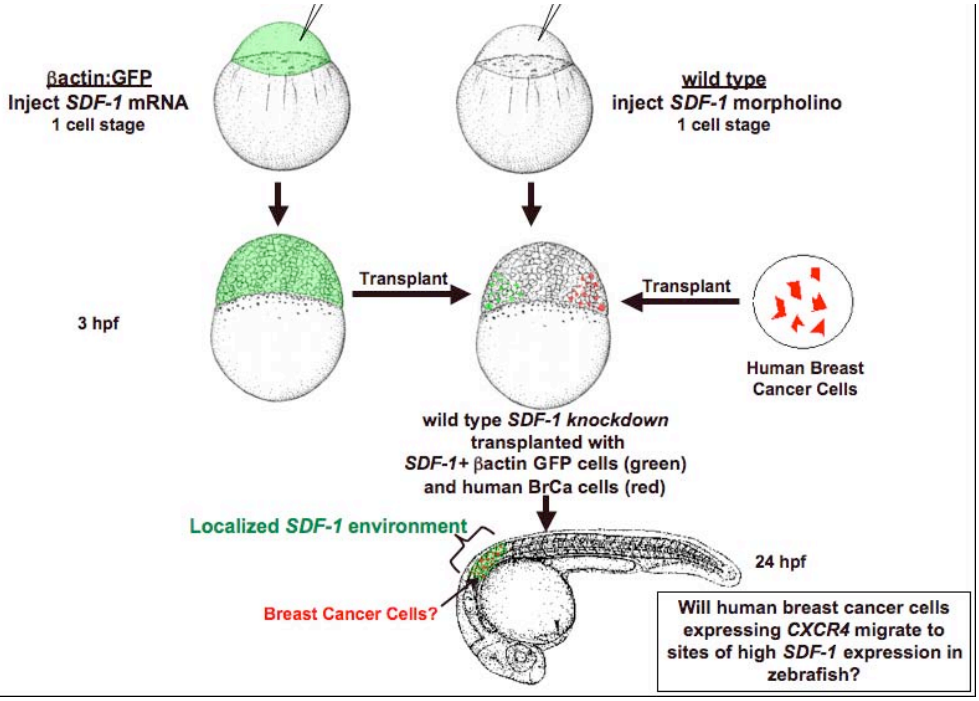


**Figure 5. Planed experimental scheme to investigate CXCR4b-dependent breast cancer cell migration *in vivo*.**

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**Appendices:**

None